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Mobilized peripheral blood progenitor cell transplant, used to reconstitute hematopoiesis following high-dose chemotherapy in breast cancer patients, is associated with a requisite period of profound thrombocytopenia. To prevent or reduce the need for repeated platelet transfusions, we designed a preclinical study to expand sufficient numbers of megakaryocyte progenitors and differentiated MK to be used as an autologous supplement to the conventional peripheral blood autograft. Our previous results have shown that IL-3 added to thrombopoietin produced over three-fold more megakaryocytes than with TPO alone. In view of the unavailability of interleukin-3 (IL-3) for clinical use in the U.S., the use of Promegapoietin, a chimeric cytokine with dual activation of thrombopoietin and IL-3 receptors, in our earlier study appeared promising. We were able to confim that Promegapoietin is indeed the cytokine of choice for megakaryocyte production. Using it at a concentration of 200 ng/ml in a serum-free medium enabled us to harvest 6.4 megakaryocytes per peripheral blood CD34+ cell seeded. Bone marrow cells were twice as productive, and umbilical cord blood 8.5-fold more productive as peripheral blood. These results will enable us in a short interval of time to start a clinical trial for testing the effectiveness of autologous megakaryocyte transplants for supportive care of breast cancer patients.

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Joan Cohen 9.29.98

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INTRODUCTION

The use of autologous peripheral blood progenitor cell (PB) transplant, following high dose chemotherapy in the treatment of breast cancer patients, results in platelet recovery which usually lags behind the relatively rapid neutrophil recovery (1-4). To prevent or reduce the need for repeated platelet transfusions, we designed a preclinical study to expand sufficient numbers of megakaryocyte (MK) progenitors and differentiated MK to be used as an autologous supplement to the conventional peripheral blood autograft. We had initially proposed to culture mobilized peripheral blood progenitor cells from patients randomized to receive either GM-CSF or PIXY321 and to compare the results with culture of isolated progenitor cells from bone marrow (BM) and umbilical cord blood (UC).

Midway through the first year, we substituted thrombopoietin (TPO), a cytokine which had just been cloned and thought to be specific for the megakaryocyte (MK) lineage, for serum from aplastic patients, known to contain a MK-stimulating factor. The advantage of using TPO, a serum-free cytokine, was compounded by its effectiveness in obtaining considerable concentrations of cultured MK. Mononucleated and CD34+ bone marrow cell cultures indicated that TPO/IL-3 was the most efficient cytokine combination for megakaryocyte production (5). Since most patients enrolled in the clinical trial comparing mobilization of GM-CSF vs. PIXY321 had low levels of CD34+ cells, cultures were carried out with mononucleated cells.

In year 02, the study comparing the effectiveness of GM-CSF and PIXY321 in priming MK production in cultures of mononucleated cells from PB harvests was completed. The results showed that the number of MK per CD34+ cells, was greater in PIXY321- than in GM-CSF-mobilized samples. However, since the frequency of CD34+ cells was greater in GM-CSF- than in PIXY321-mobilized samples, there was no significant difference in the overall absolute number of MK produced per mononucleated cell between PIXY321 and GM-CSF-mobilized samples (6). By the end of year 02, we were notified by Immunex Corporation that PIXY321 would no longer be available. We therefore revised our Statement of Work to the effect of using progenitor cell samples from patients mobilized with either Synthokine/G-CSF or G-CSF or GM-CSF. In the process of optimizing MK production, we discovered that a soluble factor from BM stroma enhanced the TPO-stimulated megakaryocyte production. Such a factor could potentially be used in our cultures.

During year 03, we attempted to characterize the MK-stimulating factor from BM stroma. The same stimulation was obtained with BM stroma conditioned medium but we were not able to identify this soluble factor. It was neither accounted for by stromal TPO, IL-3, IL-6, SCF and heparan sulfate activities, nor modified by physical contact. The main information gained from this investigation, relative to the overall objectives of our proposal, pertains to the possible use of autologous stromal conditioned medium, in addition to TPO/IL-3, to enhance MK production. In view of the FDA removal of IL-3 for clinical use, we had to find a substitute for this cytokine. It was fortuitous that Searle R&D developed Promegapoietin (PMP), a chimeric cytokine with dual activation of TPO and IL-3 receptors. Our preliminary results had indicated that PMP can indeed substitute for TPO/IL-3 for stimulating MK growth. Moreover, in view of the possible unavailability of autologous plasma to supplement our cultures, we were able to find an efficient commercially-available serum-free medium for growing MK.

In this final year, we were able to confirm that PMP is the cytokine of choice for the expansion of both differentiated MKs and MK progenitors (colony-forming unit MK, CFU-MK, and burst-forming MK, BFU-MK).

BODY

Experimental Methods:

1) Preparation of low density non-adherent mononuclear cells: BM, UC and PB samples were collected in accordance with the guidelines of the Institutional Review Board on Human Subjects. BM, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in a special anticoagulant mixture designed to prevent platelet activation and containing final concentrations of 50 U/ml preservative-free heparin, 1 mM Na_2EDTA, 1 mM adenosine, 2 mM theophylline, 2.2 μM prostaglandin E_1 and 0.1 mg/ml DNase I. Marrow cells were repeatedly extracted from bone fragments with a modified MK medium (7) which consists of Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (Dulbecco's PBS, Gibco) containing 13.6 mM Na citrate, 11 mM dextrose, 1 mM theophylline, 1 % bovine serum albumin, 2.2 μM PGE_1 and 0.1 mg/ml DNase I. Following homogenization by passage through a 18 gauge needle, low density cells were extracted with the use of Ficoll-Paque as described (8). Cells resuspended in MK medium were centrifuged at 380xg through a 10 % human serum albumin cushion in PBS to reduce platelet contamination. Residual red cells were lysed with NH_4Cl as described (9) and the remaining cells recovered by centrifugation through a 10 % human serum albumin cushion. Adherent cells were discarded following overnight incubation in Iscove Modified Dulbecco's medium (IMDM) containing 10 % fetal bovine serum (FBS). All culture media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 4 μg/ml gentamycin, and incubation carried out at 37°C in a 5 % CO_2 fully humidified atmosphere.

UC was collected in the special anticoagulant. Buffy coat and red cells were submitted to Ficoll separation and the remaining steps carried out as for bone marrow cells. Frozen PB were rapidly thawed at 37°C and added to thawing medium consisting of IMDM, 25% FBS, 12.5 U/ml heparin and 1.25 mg% DNase I. Residual red cells were lyzed, and remaining MNCs were washed and treated as described above for the elimination of adherent cells.

2) Purification of CD34+ cells: CD34+ cells were purified by positive selection using the CD34 magnetic cell sorting Mini-MACS kit (Myltenyi Biotec, Auburn, CA) in accordance with the manufacturer's recommendation. A recovery of about 60 % CD34+ cells was obtained with a purity of 86.4 % ± 1.5 S.E.M (n=6) on the basis of flow cytometric analysis following staining with PE-anti-CD34 (HPCA-2).

3) Culture conditions: Purified CD34+ cells were cultured for 12-14 days at 37°C at a seeding concentration of $5x10^4$ cells/ml in X-Vivo-20 (Biowhittaker, Walkersville, MD) a serum-free medium described in the Results section to be efficient for growing human MK. In a few experiments, results were compared with using seeding densities of $1x10^5$ and $4x10^5$ cells/ml. TPO (Zymogenetics Corp.) was used at a concentration of 50 U/ml (10 U = TPO quantity which stimulates one-half maximal proliferation of BaF3/mpl cells) which yielded the maximal concentration of MKs. Concentrations of IL-3 (R&D Systems, Minneapolis, MN) and Promegapoietin (PMP, Searle R&D), a chimeric dual agonist of c-Mpl and IL-3 receptors, were 10 ng/ml (0.4-1.6 units based on 1 unit=ED50 of TF1 cell line proliferation) and 200 ng/ml, respectively, and were optimal for MK growth. In a few experiments, Progenipoietin (ProGP, Searle R&D), a chimeric dual agonist of the G-CSF and Flt-3 receptors was used at a concentration of 200 ng/ml.

4) Cell labeling and flow cytometric analysis: The relative frequency of megakaryocyte progenitors was determined on purified CD34+ cells. Cell aliquots were treated with 5 mM EDTA which was present throughout the staining procedure. This treatment detaches most platelets and platelet fragments from cells which otherwise would stain with the anti-CD41 antibody (anti-GPIIb).

After washing, the cells were double-stained with phycoerythrin (PE) conjugated-anti-CD34 (HPCA-2, Becton-Dickinson) and fluorescein isothiocyanate (FITC) conjugated anti-CD41 (Immunotech-Amac, Westbrook, ME) and analyzed by flow cytometry. Negative controls were PE-anti-mouse IgG_1 and FITC-anti-mouse IgG_1 used at equivalent IgG_1 concentrations. The relative frequency of mature MKs was determined by flow cytometric analysis of cells stained with FITC-anti-CD41. Flow cytometric analysis was performed using a Coulter ELITE dual laser flow cytometer. Fluorescence attributable to FITC- and PE-labeled antibodies was determined using excitation by an argon laser operating at 488 nm and adjusted to 0.3 W. Emission from fluorescein and PE was measured using band pass filters of 530 ± 15 nm and 575 ± 15 nm, respectively. The per cent positive cells was calculated by substracting the percent positive of the isotype control within the same integration region.

5) Clonogenic Assay: Clonogenic assays were carried out using a serum-free collagen assay (Easyclone MegaClonogenic Assay, Hemeris, or MegaCultC, Stem Cell Technologies, Vancouver) according to the manufacturer's instructions. The cytokines used were TPO, 50 U/ml, and/or IL-3, 10 ng/ml; or PMP, 200 ng/ml. CFU(colony-forming unit)-MK were scored after 8 days, and BFU (burst-forming unit)-MK after 14 days following fixation with methanol:acetone (1:1) and immunostaining with a-GPIb and a-GPIIb (Immunotech, Westbrook, ME). Antibody binding was revealed with an APAAP kit (Dako).

6) Statistical analysis: Comparisons between CD34+ cell sources, cytokines, and media were done using the two tailed student t-test. Samples within the same cell sources were compared using the paired t-test, and samples from different cell sources were compared using the unpaired t-test. Statistically significant results were those comparisons that had p-values less than 0.05.

Results:

MEGAKARYOCYTE EX VIVO EXPANSION POTENTIAL OF THREE HEMATOPOIETIC SOURCES IN SERUM AND SERUM-FREE MEDIUM

During this last year, we have optimized our ex vivo expansion protocol and were able to confirm the preliminary results already reported. The PB used in our investigation were mostly from patients mobilized with G-CSF. A few patients were mobilized with G-CSF and synthokine.

Evaluation of culture media: BM CD34+ cells were cultured with 200 ng/ml PMP in serum-free medium and total production of MKs was compared to that obtained by culturing cells from the same donor in IMDM with 2.5% human serum . The greatest MK production was obtained with the use of serum-free medium (Easymega, Hemeris, Sassenage, Fig. 1). Compared to human serum-containing medium, incubation in serum-free medium resulted in increased MK frequency and cell proliferation. Similar efficiency was obtained with X-vivo-20 (Biowhittaker, Walkersville, MD) which is presently used routinely in our laboratory.

Cell proliferation: UC-derived CD34+ cells showed greater total cell proliferation than either bone marrow or peripheral blood for all cytokines tested, in both serum-containing and serum-free medium, while there were no differences between BM and PB (Figure 2a & b). In serum-containing medium, IL-3 induced greater cell proliferation than TPO for BM and PB, but not UC, and combining TPO with IL-3 led to a cooperative increase in cell proliferation compared to TPO alone and IL-3 alone (Figure 2a). In the same culture conditions containing UC, the

proliferative effect of PMP or TPO+IL-3 was additive compared to TPO or IL-3 alone. For UC, TPO+IL-3 induced greater cell proliferation than PMP in the presence of serum (Figure 2a), while the inverse was true in its absence (Figure 2b).

Megakaryocyte progenitors: There were no differences between TPO+IL-3 and PMP in the number of either CFU-MK or BFU-MK colonies produced from any cell source (Table 1). Both PMP and TPO+IL-3 samples showed an additive effect when compared to samples exposed to TPO or IL-3 alone in relation to the number of CFU-MK and BFU-MK (BM data not shown). UC CD34+ cells produced more CFU-MK and BFU-MK colonies than BM or PB (Table 1). The size of BFU-MK from UC (>200 cells per colony) was larger than the equivalent colonies from BM or PB (data not shown). In separate experiments, the frequency of MK progenitors (CD34+/CD41+) in the initial CD34+ cell population was measured by flow cytometry. CD34+/CD41+ cell frequency was greater for BM than for the other two hematopoietic sources (Table 2). There was no difference between UC and PB CD34+/CD41+ cell frequency. When the ratio of CFU-MK or BFU-MK colonies per seeded CD34+/CD41+ cell was calculated, UC produced a higher ratio than either BM or PB (Table 2).

Relative frequency and total megakaryocyte production: We were able to confirm with additional experiments that the percentage of CD41+ cells was greater in PMP, TPO, and TPO+IL-3-supplemented cultures when compared to IL-3-supplemented cultures in serum-containing medium . In these culture conditions, PMP induced a lower CD41+ cell frequency than TPO alone. When BM were cultured in serum-free medium, TPO induced lower CD41+ cell frequencies than either TPO+IL-3 or PMP. Cultured BM and UC exhibited higher CD41+ cell frequencies than PB for all cytokine and culture medium combinations. Overall, the MK harvest was 6.4 MK/PB CD34+ cell; BM was twice as productive and UC was 8.5-fold more productive as PB. These results show that serum-free medium is at least as efficient as serum-containing medium for generating a high percentage of MK in cultures involving the three hematopoietic sources.

UC produced greater numbers of MKs than BM or PB under all conditions and BM produced more MK than PB. The addition of IL-3 to TPO cooperatively increased MK production for BM and PB compared to using TPO or IL-3 alone. The same cooperativity was seen with PMP. For UC in the presence of serum, the effect of TPO+IL-3 or PMP was additive compared to TPO or IL-3 alone. While higher numbers of UC MK were obtained with TPO+IL-3 when compared to PMP in the presence of serum, the inverse occurred in its absence. Overall, using either TPO+IL-3 or PMP resulted in greater MK production compared to TPO or IL-3 alone.

Effect of high seeding density and additional progenipoietin: Preliminary experiments showed that using $4x10^5$ seeding density of PB CD34+ cells to minimize culture volumes had no deleterious effect on culture performance, and using additional progenipoietin (ProGP) enhances even further MK expansion.

TIME TO PLATELET ENGRAFTMENT

Since the close of the PIXY321 vs. GM-CSF clinical trial, we have continued to collect long-term follow-up on those patients, whether or not their specimens were studied *in vitro* for their capacity to generate MK. A manuscript summarizing that data is in preparation. In our publication, *"In vitro* production of megakaryocytes from PIXY321 vs. GM-CSF primed peripheral blood progenitor

cells" (6), we demonstrated that eighteen Northwestern Memorial patients randomized to receive either PIXY321 or GM-CSF, PIXY321-mobilized samples produced more MK per CD34+ cell than GM-CSF-mobilized samples. This is the first data to suggest that the particular cytokine used for mobilizaion determines the lineage commitment of the CD34+ cells collected. Whereas Immunex withdrew PIXY321 from clinical trial, we have subsequently used specimens from patients with breast cancer mobilized with any cytokine or cytokine combination, including those apheresed during recovery from myelosuppressive chemotherapy for our *in vitro* studies. Unlike the patients entered on the PIXY321 vs. GM-CSF trial, these patients represent a heterogeneous group, consisting of both heavily pretreated and minimally pretreated individuals receiving different cytokine combinations (G-CSF alone, G-CSF plus Daniplestim, Myelopoietin) and different treatment regimens. Higher doses of G-CSF were used in cases that were expected to be poor mobilizers. In addition, according to our most recent protocols, aphereses from some of our breast cancer patients undergo CD34+-cell selection using the Isolex device (Baxter). The heterogeneous nature of this group, and small numbers in each subset have made it statistically unreasonable to look at correlations between MK generation and time to platelet engraftment in these cases. Instead, we have focused on optimizing methods for expanding MK for use in supporting patients through the obligate period of thrombocytopenia associated with high-dose chemotherapy. The generation of MK using our optimized methodology from PB, BM and UC has been compared, and this data has been submitted for publication (see above).

CONCLUSIONS

In this fourth and final year we completed our study with PMP with additional cultures of progenitor cells from PB, BM and UC which allowed us to assess the statistical significance of our results. Although peripheral blood was less productive than either umbilical cord blood or bone marrow, enough MK could be generated from peripheral blood for transfusion purposes. Using a cocktail of seven cytokines, including MIP-1 α, IL-3, IL-6, IL-11, Flt-3L, SCF and MGDF, in their *ex vivo* expansion protocol, Bertolini et al. (10) transplanted an enriched MK product into patients (10). Two of the four patients who received the highest megakaryocyte infusions (2.5-21.3 x 10^5 CD61+ cells/kg) did not require platelet transfusions. Our goal is to transplant a minimum of 21.3 x 10^5 MK/kg patient. While Bertolini et al (10) were able to harvest an average of 4.1 MK per cell seeded, we have shown that PB CD34+ cells cultured in serum-free medium with a single chimeric cytokine, PMP, will generate an average of 6.4 MK per cell seeded. Based on these figures, for a 70 kg patient, 2.3 x 10^7 CD34+ cells should be cultured for 12 days to reach our goal, well within technical and clinical limits. BM cells were twice as productive, and UC cells 8.5-fold more productive as PB, further reducing the starting material required. Upon confirmation of our results pertaining to the additional use of ProGP and high seeding density, this goal will be even more easily achieved. While reviewing our progress of the past four years, it is apparent that we have acquired expertise in *ex vivo* expanding MK and will be soon poised to start a clinical trial.

The relationship between MK content of PB autografts and platelet engraftment still remains to be determined. Recent literature indicates that the number of infused CD34+/CD41+ cells may be a more powerful predictor of platelet recovery than CD34+ cell content alone, suggesting that the flow cytometric analysis of the MK marker glycoprotein IIb/IIIa on CD34+ cells provides the most accurate indication of the platelet reconstitutive capacity of a PB autograft (11). In another recent

publication, investigators correlating the results of assays for MK progenitors (CFU-MK) with time to platelet recovery found that the predictive value of the CFU-GM assay was equivalent to that of CFU-MK assay (2). Hence, to follow-up on our finding (6) regarding lineage commitment of mobilized CD34+ cells, we have initiated a phase III trial within the Eastern Cooperative Oncology Group (ECOG)(E1Y98). A larger number of patients than available at a single institution will be accrued in a short time interval. In this trial, the combination of a synthetic IL-3 receptor agonist and G-CSF will be compared to G-CSF alone for purposes of mobilizing peripheral blood progenitor cells. The combination of the IL-3 receptor agonist and G-CSF is expected to have benefits similar to those seen with PIXY321, a bifunctional molecule binding both the IL-3 and GM-CSF receptors. In this study, all aphereses will be studied for MK progenitors using both clonogenic assays and flow cytometry. We will investigate an association between the results of these *in vitro* studies and platelet recovery and the numbers of days of platelet transfusions. This study will be supported by ECOG and Searle.

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APPENDIX: Figures and Tables

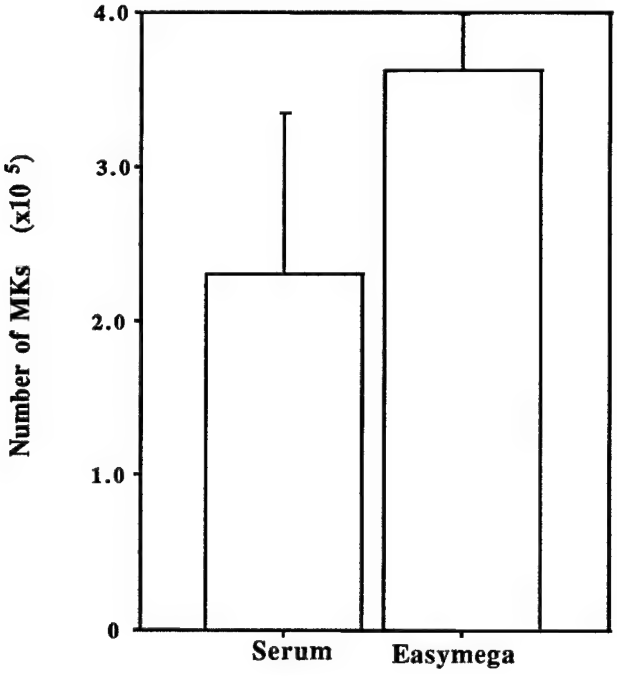

Figure 1: MK productivity in the presence of serum-free medium. MK productivity was calculated on the basis of the relative frequency of MK and the total number of Trypan Blue negative cells. Using PMP, serum-free medium (Easymega) produced more MK than serum-containing medium.

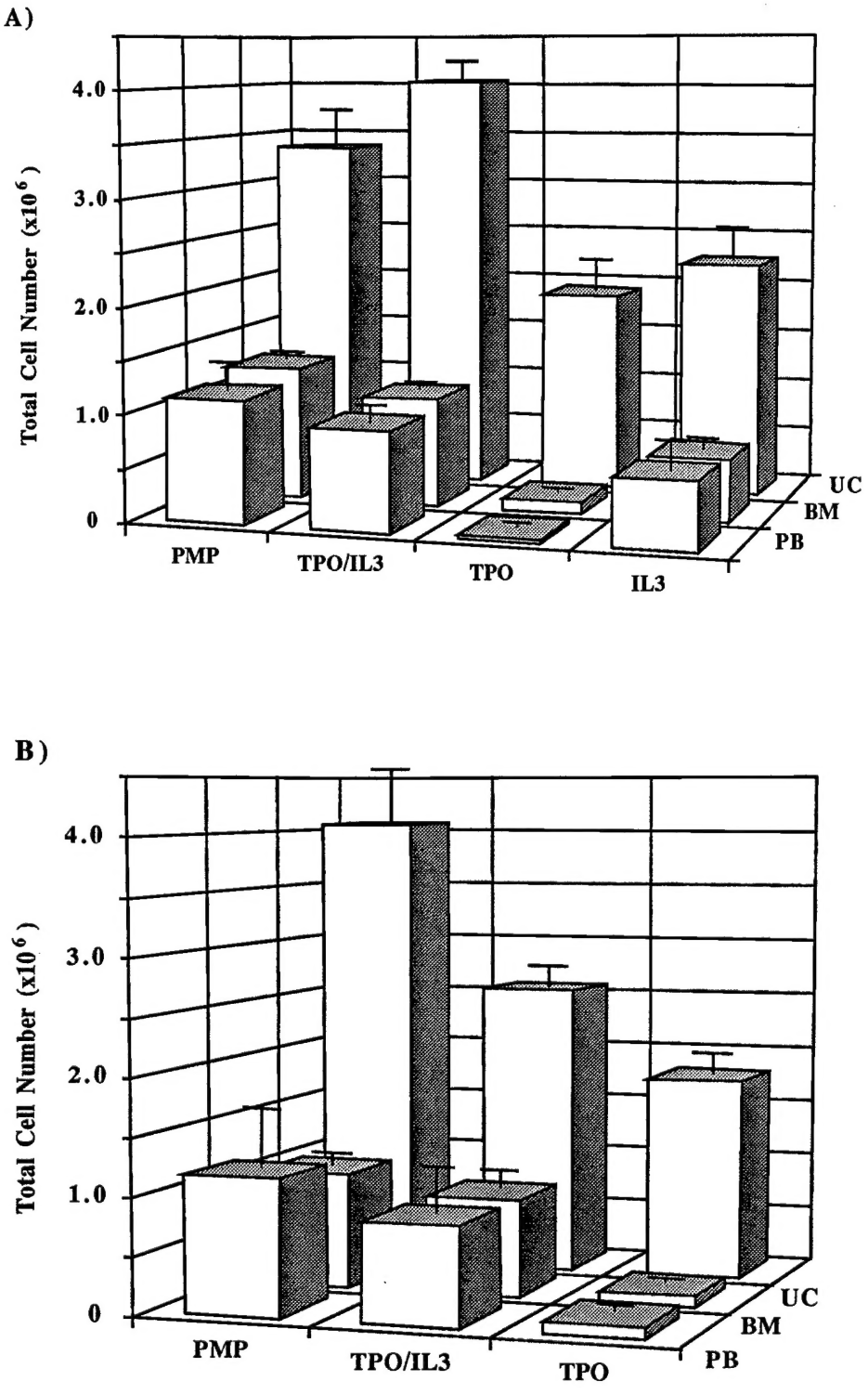

Figure 2: Total cell proliferation. The number of Trypan Blue-negative cells per ml of culture was counted for each sample.The mean±SEM total cell proliferation is shown. UC, UC; BM, BM; PB, PB. (A) Cells cultured in serum-containing medium; (B) Cells cultured in serum-free medium.

Table 1: Clonogenic Capacity of CD34+ Cells*

Cell Source	CFU-MK		BFU-MK	
	PMP	TPO/IL-3	PMP	TPO/IL-3
UC (n=5)	157±26	149±13	133±39	109±20
BM (n=5)	84±29	88±23	47±11	39±10
PB (n=3)	27±4	32±2	12±2	11±1

*Total number of colonies measured ± SEM. Each sample was analyzed in duplicate.

Table 2: Megakaryocyte Colonies Derived from MK Progenitors

Cell Source	%CD34+/41+ ±SEM (n)	CFU-MK per CD34+/41+ Cell ±SEM (n)	BFU-MK per CD34+/41+ Cell ±SEM (n)
UC	1.8% ± 1.1% (22)	4.5±0.7 (18)	3.1±0.4 (18)
BM	4.0% ± 1.8% (19)	1.0±0.1 (26)	0.4±0.1 (21)
PB	1.6% ± 1.5% (23)	1.6±0.3 (20)	0.7±0.2 (12)

Results are for colonies cultured with TPO+IL-3. Percent $CD34^+/41^+$ determined by flow cytometry. CFU- and BFU-MK per $CD34^+/41^+$ cell was calcultated by dividing the number of colonies by the number of $CD34^+/41^+$ cells seeded.

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Dr. Jane N. Winter, coPI

Dr. Mirta Schattner for years 1-3 and Dr. Yuru Meng for year 4 as Post-Doctoral Fellows

Phil Lefebvre, Research Technologist/Graduate Student

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